Inhibitory Effect With Antisense Mitogen-Activated Protein Kinase Oligodeoxynucleotide Against Cerebral Vasospasm in Rats

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Background and Purpose—Mitogen-activated protein kinase (MAPK) may be associated with the pathogenesis of cerebral vasospasm after subarachnoid hemorrhage (SAH). This study aimed to clarify the role of MAPK expression and activation during cerebral vasospasm and to evaluate the therapeutic effect on cerebral vasospasm using an antisense MAPK oligodeoxynucleotide (ODN).

Methods—Antisense MAPK, sense MAPK, or scrambled ODN was injected into the rats intracisternally. We used a single-hemorrhage experimental SAH model to assess vasospasm in the basilar arteries at 30 minutes, 1 day, and 2 days after SAH by cross-sectional area measurement and other histological parameters. Immunohistochemistry and Western blot analysis were used to quantify MAPK expression and activation. In addition, a double-hemorrhage rat SAH model was used to test the effect of post-SAH treatment with antisense MAPK ODN.

Results—Antisense MAPK therapy significantly inhibited cerebral vasospasm when compared with sense MAPK or scrambled ODN treatment on day 2. The immunohistochemistry and Western blotting performed in the basilar artery of rats that received antisense MAPK ODN demonstrated inhibition of MAPK and phosphorylated MAPK on day 2. In post-SAH treatment study, antisense ODN reduced MAPK and phosphorylated MAPK in the basilar artery and attenuated cerebral vasospasm.

Conclusions—MAPK activation, but not expression, might be implicated with sustained smooth muscle contraction during cerebral vasospasm after SAH. This study suggests that antisense MAPK ODN strategy is an effective treatment against cerebral vasospasm.

Key Words: mitogen-activated protein kinases ■ oligonucleotides, antisense ■ vasospasm, intracranial ■ rats

Cerebral vasospasm greatly influences morbidity and mortality in patients after subarachnoid hemorrhage (SAH). Although many investigators have researched the signal transduction pathways leading to cerebral vasospasm, the pathogenesis remains speculative. Mitogen-activated protein kinase (MAPK) is one of the signaling pathways for sustained vascular smooth muscle contraction. Recently, our laboratory suggested that activating the MAPK signaling pathway is associated with the cerebral vasospasm. MAPK is a family of serine/threonine protein kinases involved in cell growth and differentiation. The critical isoforms in this signal pathways are the 44-kDa MAPK and 42-kDa MAPK, alternatively termed extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), respectively. MAPKs (ERK1 and ERK2) are activated by the phosphorylation of both tyrosine and threonine residues and in turn induce smooth muscle contraction by phosphorylating caldesmon.

We previously examined the effects of MAPK inhibitors such as PD-98059 and U-0126 on cerebral vasospasm in an animal experiment. Although the MAPK inhibitors demonstrated inhibitory effects on cerebral vasospasm after SAH, the inhibitory effects were inadequate. Recently, several authors reported that directly injecting endothelin or heme oxygenase-1 antisense ODN into the subarachnoid space prevented cerebral vasospasm. In addition, phosphorothioate-modified oligodeoxynucleotide (ODN) antisense strategy has been reported as a treatment modality that inhibits MAPK. In this study, we attempted to clarify the role of MAPK on cerebral vasospasm after SAH using antisense MAPK ODN.

Subjects and Methods

Experimental Design

Male Sprague-Dawley rats (n=64), each weighing from 260 to 340 g, were randomly assigned to 3 groups. Rats received antisense MAPK ODN (ASOD group, n=21), sense MAPK ODN (SOD group, n=20), or scrambled ODN (SCOD group, n=23) intracisternally. All animals were subjected to single SAH 24 hours after administration of ODNs (antisense MAPK, sense MAPK, and...
scrambled ODN). Nine (14%) of 64 rats died within a few minutes after injection of blood, and the data of these rats were excluded. Twenty male Sprague-Dawley rats, each weighing from 260 to 340 g, were subjected to double SAH to examine the effect of post-SAH treatment with antisense MAPK ODN. The animals were divided into 2 groups after SAH: double-SAH group (only double SAH with no treatment, n=10) and double-SAH + ASOD group (double SAH with antisense MAPK ODN, n=10). Mortality from SAH was 20%. An additional 12 rats were euthanized as a control group. All procedures were approved by the Animal Care and Use Committee at the University of Mississippi Medical Center.

Preparation of ODNs
The sequences for ODNs used in this study were 5'–GCCGCGCCCGCGCCG-3' (antisense ODN) (at 41 to 25, Gen-Bank accession no. X65198), 5'–ATGGCGGGC GCCGCCG-3' (sense ODN), and 5'–CGCCGCTGGCGGACCC-3' (scrambled ODN). The ODNs were prepared with phosphorothioate modification. The dosage was selected according to the method of Onoda et al.10 Briefly, the 10-nmol ODNs were diluted in 100 μL saline to a concentration of 100 μmol/L. After the animals were anesthetized, 100 μL ODN was administered into the cisterna magna 24 hours before single SAH or immediately after double SAH as described below. Because the total cerebrospinal fluid (CSF) in a rat is approximately 1 mL, the ODNs were ultimately diluted to 10 μmol/L.

ODN Administration and Induction of SAH
Prior ODN Administration on Single-Hemorrhage Vasospasm Model
After the rats were anesthetized intraperitoneally with ketamine hydrochloride (70 mg/kg) and xylazine (7 mg/kg), they were weighed. The subjects were placed on an electric heating pad during surgery to maintain rectal temperature at approximately 37°C. The subjects were placed in the prone position, and a skin incision was made along the midline in the neck. After the occipital bone was cleared of muscular attachment, the occipito-atlantal membrane was exposed for insertion of a 25-gauge needle into the cisterna magna through the arachnoid membrane. After withdrawal of 0.1 mL CSF, 100 μL ODN was administered into the cisterna magna, and the first surgical phase was finished. Twenty-four hours later, animals were anesthetized in the same manner, and the second surgical phase was started. After the right femoral artery was opened, we inserted a 27-gauge needle and withdrew 1 mL autologous blood from the artery. The subjects were turned in a prone position, and the occipito-atlantal membrane was exposed within 1 minute. After withdrawal of 0.1 mL CSF, 0.35 mL autologous nonheparinized blood was infused over 3 minutes. Anesthesia was maintained by repeated injections of ketamine hydrochloride and xylazine as needed. Surgery concluded with the rats being placed head down for 30 minutes to pool the blood around the brain stem.

Post ODN Administration on Double-Hemorrhage Vasospasm Model
In the double-SAH model, we injected 0.35 mL autologous nonheparinized blood in the first surgical phase. Then the rats were placed head down for 30 minutes. In the second phase, the rats received a second blood injection 2 days after the first injection in the same procedure, immediately followed by 100 μL antisense ODN administration. Surgery concluded with the rats being placed head down for 30 minutes again.

Histological Examination and Cross-sectional Area Measurement
The single-hemorrhage animals were euthanized at the following time points: at 30 minutes (n=3 in each group), on day 1 (n=4 in each group), and on day 2 (n=7 in ASOD group, n=8 in SEOD group, and n=7 in SCOD group) after SAH. The double-hemorrhage rats were killed on day 7 (n=5 in double-SAH group and n=5 in double-SAH + ASOD group). Furthermore, normal rats were killed as a control group (n=5). After perfusion and transcardial fixation with 100 mL PBS and 200 mL 4% paraformaldehyde, the brains were removed and stored in the fixative at 4°C. Perfusion was performed at a standard height of 100 cm from the chest (100 mm Hg). Sections were embedded in paraffin and sliced into 5 μm. Then they were stained with hematoxylin and eosin.

The cross-sectional area of basilar artery was calculated as described previously.11,12 The basilar arteries were transected at the same point: two thirds of the distance from the proximal side to avoid the arterial branches. These sections were prepared over 200 μm. Three cross-sections of each vessel were selected randomly for measurement. Using a digital imaging system (Pentium base computer, digital camera, microscope, and Metamorph software), we measured the actual circumference of the vessel lumen and calculated the r value (r=measured circumference/2π) to correct for any vessel deformation. Based on the calculated r value, we calculated the area of a generalized circle (πr²) and then averaged the resultant values.

Immunohistochemistry
After deparaffinization, using the Vectastain Elite ABC kit (Vector Laboratories), we performed immunohistochemical staining to demonstrate MAPKs (n=14 in ASOD group, n=15 in SEOD group, and n=14 in SCOD group) after SAH. The double-hemorrhage rats were killed on day 7 in control group) or monoclonal

Tissue Preparation for Western Blot
Two days after the onset of single SAH, the rats (n=4 in ASOD, SEOD, and SCOD groups) were deeply anesthetized with isoflurane and euthanized by exsanguination. In the case of double SAH, the rats (n=3 in double-SAH group and n=3 in double-SAH + ASOD group) were killed on day 7. An additional 7 rats were euthanized as a control group. The basilar arteries were removed from the brain stems and immediately frozen in liquid nitrogen. Frozen tissue was added to lysis buffer (PBS pH 7.4, 1% Igepal CA-630, 0.1% SDS) containing protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 0.15 μmol/L aprotinin, 2.1 μmol/L leupeptin) and then homogenized with an ultrasonic wave (3 times for 10 seconds each). After incubation with 30 μL phenylmethylsulfonyl fluoride (0.057 mol/L) for 30 minutes following centrifugation, the supernatant was collected. Two microliters of lysate was taken for Bio-Rad protein assay, and the remaining was frozen and stored at −80°C for Western blotting.

Western Blotting
After the lysate was mixed with 2X sample buffer (62.5 mmol/L Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 50 mmol/L dithiothreitol, 0.1% bromphenol blue, and deionized water), 20 μg protein was boiled for 5 minutes. Each sample was run on 10% polyacrylamide gels with 4% stacking gel (SDS-PAGE) for 2 hours and then transferred to polyvinyl difluoride membrane for 1.5 hours. The membrane was blocked with 5% nonfat dry milk in TBST (0.1% Tween-20, 150 mmol/L NaCl, and 20 mmol/L Tris base pH 7.6) for 1 hour at room temperature and washed in TBST 3 times for 5 minutes each. The membrane was incubated with either monoclonal mouse anti-ERK1 + ERK2 antibody (1:2000; Zymed Laboratories) (n=4 in ASOD, SEOD, and SCOD groups; n=3 in double-SAH and double-SAH + ASOD groups; n=7 in control group) or monoclonal
mouse anti-phospho-ERK1 + ERK2 antibody (1:2000; New England BioLabs) (n=3 in ASOD, SEOD, and SCOD groups; n=3 in double-SAH and double-SAH + ASOD groups; n=6 in control group) in blocking solution overnight at 4°C. After washing with TBST, the membrane was incubated with peroxidase-conjugated goat anti-mouse secondary antibody (1:2000) for 1 hour at room temperature. Finally, the membrane was incubated with ECL detection reagent (Amersham Pharmacia Biotech) for 5 minutes and then exposed to x-ray film. Immunoblotting for β-actin was performed with monoclonal mouse anti-β-actin antibody (1:5000; Sigma Chemical Co) as an internal control because the level of β-actin does not change with SAH.17,18 The density ratios of the ERK1, ERK2, and phospho-ERK2 protein bands to β-actin band were analyzed using Quantity One software (Bio-Rad Laboratories). The level of MAPK/β-actin or phospho-MAPK/β-actin in the ASOD, SEOD, and SCOD groups was expressed as a percentage of the level in the control group.

Statistical Analysis
All data were expressed as the mean±SEM. Statistical comparisons between 2 groups were evaluated using an unpaired t test. We used the one-way ANOVA followed by Scheffé’s F post-hoc test to perform statistical comparisons among 3 or more groups. A probability value was significant at P<0.05.

Results
Histological Examination
At the time points of 30 minutes and day 1, the internal elastic lamina (IEL) corrugation and the thickened media were severe (data not shown) in all 3 treated groups (ASOD, SEOD, and SCOD groups). In contrast, the IEL and media of the ASOD group were less corrugated and thickened, respectively, than those of the SEOD and SCOD groups on day 2 (Figure 1B). SEOD and SCOD groups demonstrated corrugated IEL and thickened media on day 2 (Figure 1C and 1D).

In the double-hemorrhage model, the double-SAH group showed severe corrugation of IEL and thickness of the media on day 7 (Figure 1E). In the double-SAH + ASOD group, less histological vasospasm was observed (Figure 1F).

Measurement of the Cross-sectional Area
The group values of the cross-sectional areas in the basilar artery at each time point are shown in Figure 2. Rats of 3 groups (ASOD, SEOD, and SCOD groups) demonstrated severe vasospasm at 30 minutes and mild vasospasm on day 1 after SAH, respectively, when compared with those of the control group. No statistically significant differences among the 3 groups were revealed (P>0.05, ANOVA).

By contrast, the ASOD group exhibited a statistically significant increase (P<0.05, ANOVA) of the basilar artery area on day 2 compared with the SEOD and SCOD groups. The ratios of the basilar artery areas in the ASOD, SEOD, and SCOD groups to that in the control group were 99%, 65%, and 69%, respectively. The time course of arterial narrowing after SAH in the SEOD and SCOD groups was similar to that in previous studies.12,19,20 In addition, the ASOD group did not differ significantly from the control group on day 2 (P>0.05, ANOVA).

In the double-hemorrhage model, the double-SAH group showed severe vasospasm on day 7 (44%). Post-SAH treatment of antisense MAPK ODN induced a marked attenuation of cerebral vasospasm (86%). There was a statistically significant difference between the 2 groups (Figure 3).

Immunohistochemistry for MAPK Expression
The level of MAPK expression was investigated by immunohistochemistry. Immunopositive stained cells were detected in 3 groups at 30 minutes and on day 1 after SAH (ASOD, SEOD, and SCOD groups) (Figure 4A, 4B, 4D, 4E, 4G, 4H). On day 2, the ASOD group (Figure 4C) exhibited weaker MAPK expression than the SEOD (Figure 4F) or the SCOD groups (Figure 4I). MAPK immunoreactivity in the
The ASOD group was also weakest on day 2 (Figure 4C) at all time points (Figure 4A through 4C). MAPK immunoreactivity was mainly localized in the cytoplasm and partially in the nucleus of the smooth muscle cells. There were no immunopositive cells in the negative controls.

**Western Blot Analysis for MAPK Expression and Phosphorylation**

Immunoblotting showed the depletion of MAPK protein in the ASOD group on day 2 (Figure 5A). Antisense ODN reduced MAPK expression by 65% (Figure 5B). In contrast, there was no statistical significance between SCOD and control groups. Although the SEOD group exhibited increased MAPK expression registering 43% more than the control group, the comparison did not demonstrate statistical significance ($P>0.05$, ANOVA). In post-SAH treatment study, antisense ODN decreased MAPK expression by 37% on day 7. MAPK expression in the double-SAH group did not differ from that in the control group (Figure 5C and 5D).

Western Blot Analysis for MAPK Expression and Phosphorylation

MAPKs are present in vascular smooth muscle cells, and free radicals, such as a superoxide anion, activate MAPKs. MAPKs have been positioned downstream of Ras, and Lander et al. recently reported that Ras is a common signaling target of free radicals. Ras is regulated by 2 guanosine nucleotides—guanosine dinucleotide (GDP) and guanosine trinucleotide (GTP)—and is bound to GDP in an inactive state and to GTP in an active state. Therefore, increasing the GTP/GDP ratio by free radicals promotes the activation of Ras. GTP-bound Ras is bound with Raf-1 and then leads to activation (phosphorylation) of MAPK kinase (MEK) and MAPKs.

Cytokines, such as interleukin-1β or tumor necrosis factor-α, also have been shown to activate MAPK in smooth muscle cells. These cytokines lead to generating diacylglycerol, a well-known activator of protein kinase C, by stimulating a phosphatidylinositol-specific phospholipase C. Khalil and Morgan suggested that diacylglycerol activates protein kinase C, which in turn activates the MAPK signaling pathway.

Antisense Therapy for Vasospasm

Several authors have introduced an antisense strategy into the treatment of cerebral vasospasm after SAH. They succeeded in inhibiting mRNAs or proteins of the targets in

**Discussion**

**Mechanism of MAPK Activation during Vasospasm**

MAPKs are present in vascular smooth muscle cells, and free radicals, such as a superoxide anion, activate MAPKs. Antisense ODNs have been positioned downstream of Ras, and Lander et al. recently reported that Ras is a common signaling target of free radicals. Ras is regulated by 2 guanosine nucleotides—guanosine dinucleotide (GDP) and guanosine trinucleotide (GTP)—and is bound to GDP in an inactive state and to GTP in an active state. Therefore, increasing the GTP/GDP ratio by free radicals promotes the activation of Ras. GTP-bound Ras is bound with Raf-1 and then leads to activation (phosphorylation) of MAPK kinase (MEK) and MAPKs.

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Considering that free radicals or cytokines are associated with the pathogenesis of cerebral vasospasm after SAH, the mechanisms of vasospasm might be mediated by the activation of MAPK. Gerthoffer et al. and Hedges et al. have speculated that MAPK activation mediates cerebral vasospasm because MAPK activation induces the phosphorylation of caldesmon that, in turn, leads to vascular smooth muscle contraction. We have previously demonstrated that SAH could induce MAPK activation during vasospasm in an animal experimental SAH model. In the present study, MAPK activation was observed on day 2. These results are consistent with the previous finding that MAPK activation was recognized immediately and on days 2 and 7 after SAH in a canine double-hemorrhage model.

**Antisense Therapy for Vasospasm**

Several authors have introduced an antisense strategy into the treatment of cerebral vasospasm after SAH. They succeeded in inhibiting mRNAs or proteins of the targets in
the basilar artery by directly injecting antisense ODN into the cisterna magna. Onoda et al. indicated that antisense ODN in CSF could be incorporated into the cells in all layers of the arterial wall through the adventitia using labeled ODN with fluorescence. Antisense ODN is thought to bind targeted complementary mRNA sequences after transcription and to prevent translocation of the mRNA. We also observed inhibited expression of MAPK in the basilar artery on day 2 by the same method. We did not, however, observe inhibited expression of MAPK in the basilar artery at 30 minutes and on day 1. At this point, we observed different results compared with several previous studies in which antisense ODN for preproendothelin-1, heme oxygenase-1, or collagen-1 was used. In the case of endothelin-1, vascular contraction was inhibited by using antisense preproendothelin-1 ODN within 20 minutes because Clozel and Watanabe have reported that endothelin-1 is synthesized and secreted in 30 to 60 minutes after an experimental single hemorrhage in rats. Suzuki et al. and Aihara et al. have reported that other target proteins (such as heme oxygenase-1 or collagen type I) also have been synthesized comparatively early after SAH. Meanwhile, we observed no significant change in MAPK expression between the control group and the SCOD group. This observation suggests that total MAPK (MAPK expression) was not altered between normal tissues before SAH and spastic tissues within 2 days after SAH. As an explanation for this result, SAH might induce the MAPK signaling pathway by phosphorylation/activation of MAPK rather than by increasing or decreasing the level of total MAPK (MAPK expression) in the cells. MAPK expression is known to increase during the progression of cancer. On the other hand, recent reports have demonstrated that MAPK expression was not changed during ischemia or by the stimulation such as cytokines. In addition, Fuzikawa et al. documented that there was no change in ERK1 and ERK 2 expression during vasospasm in a canine SAH model. These reports support our present finding. Therefore, there might be a time-lag for the effect of antisense MAPK ODN. A possible reason is that a protein half-life for ERK1 and ERK2 is about 24 hours, and it will take antisense MAPK ODN 48 to 72 hours to inhibit the expression of MAPK.

Figure 5. Representative autoradiogram of MAPK expression and β-actin in the basilar artery on day 2 (A) and day 7 (C). Densitometric analysis of MAPK expression on day 2 (B) (n=4 in each treated group and n=4 in control group) and on day 7 (D) (n=3 in each treated group and n=3 in control group). MAPK expression was inhibited by treatment with antisense MAPK ODN. MAPK/β-actin in the ASOD, SEOD, SCOD, double-SAH, and double-SAH + ASOD groups was expressed as percentages of that in the control group. *P<0.05 compared with control group.

Figure 6. Representative autoradiogram of MAPK phosphorylation and β-actin in the basilar artery on day 2 (A) and day 7 (C). Densitometric analysis of MAPK phosphorylation on day 2 (B) (n=3 in each treated group and n=3 in control group) and on day 7 (D) (n=3 in each treated group and n=3 in control group). MAPK phosphorylation increased in SEOD, SCOD, and double-SAH groups, whereas antisense ODN prevented any significant increase in MAPK phosphorylation. Phosphorylated MAPK/β-actin in the ASOD, SEOD, SCOD, double-SAH, and double-SAH + ASOD groups was expressed as percentages of MAPK/β-actin in the control group. *P<0.05 compared with control group.
hours to deplete ERK1 and ERK2.\textsuperscript{42,43} The time-lag of this study might reflect a term for the inhibition of new MAPK synthesis. Thus, SAH might increase the activity of MAPK, rather than change the expression of MAPK, which might then play a primary role in sustaining the vasoconstruction after SAH.

We examined MAPK phosphorylation and observed that antisense MAPK ODN inhibited MAPK phosphorylation (MAPK activity), compared with sense MAPK ODN or scrambled MAPK ODN on day 2. Antisense MAPK ODN may result in a lesser degree of activation by reducing the amount of MAPK itself. Several investigators have reported that antisense MAPK ODN reduces not only MAPK expression but also MAPK activation.\textsuperscript{13,14,44} Selective MAPK inhibitors targeting an important signal transduction factor such as MEK (phosphorylates the ERK gene product).

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This study suggests that MAPK activation, but not expression, plays an important role in cerebral vasospasm after SAH. Inhibiting MAPK activation reduces cerebral vasospasm, and antisense MAPK ODN might be more effective than MAPK inhibitors.

References


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